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Attached find Appendix C for the Examiner's answer mailed 6 March 2007

ANNE KUBELIK, PH.D.
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Appendix C

Protein evolution by molecular breeding

Jeremy Minshull* and Willem PC Stemmer†

Natural evolution has guided the development of 'molecular breeding' processes used in the laboratory for the rapid modification of subgenomic sequences including single genes. The most significant recent development has been the *in vitro* permutation of natural diversity. Homologous recombination of multiple related sequences produced high-quality libraries of chimeric sequences encoding proteins with functions that differ dramatically from any of the parents. Increasingly powerful screening methods are also being developed, allowing these libraries to be screened for novel biocatalysts.

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Introduction

Enzymes are used in a wide variety of applications including food and feed processing, laundry detergents, chemicals production, paper bleaching and pharmaceutical manufacturing. The benefits of using enzymes as catalysts are that reactions can occur at moderate temperatures, toxic solvents or reactants can often be eliminated, and reactions are usually stereospecific, which is of particular benefit in the synthesis of pharmaceuticals and fine chemicals. The specificity of enzymes also obviates the need for protecting and deprotecting reactive groups, which is a source of considerable yield loss in organic syntheses.

Although three billion years of evolution have produced a wealth of protein catalysts, they are generally not optimal for a particular industrial application. While it is possible to screen enzymes from extremophiles for activity under the appropriate process reaction conditions [1,2], natural selection has selected enzymes to function in the complex mixtures of molecules within cells rather than in bioreactors. Obtaining the desired combinations of properties therefore generally requires further protein optimization.

Structural information has been used with some success to improve enzyme function [3–5]. As a general method, however, structure-based methods require time and equipment in order to generate and process very large amounts of information.

An alternative strategy to making defined changes on the basis of structural understanding is to harness the Darwinian power of recursive cycles of mutation and selection. By using directed evolution, protein engineers attempt to mimic the natural processes by which protein variants arise and are tested for 'fitness' within living systems. In this review, we will focus on the underlying rationale behind and recent advances in directed evolution, both in the methods used to generate protein variants, and in the screening strategies used to identify variants of interest.

DNA shuffling

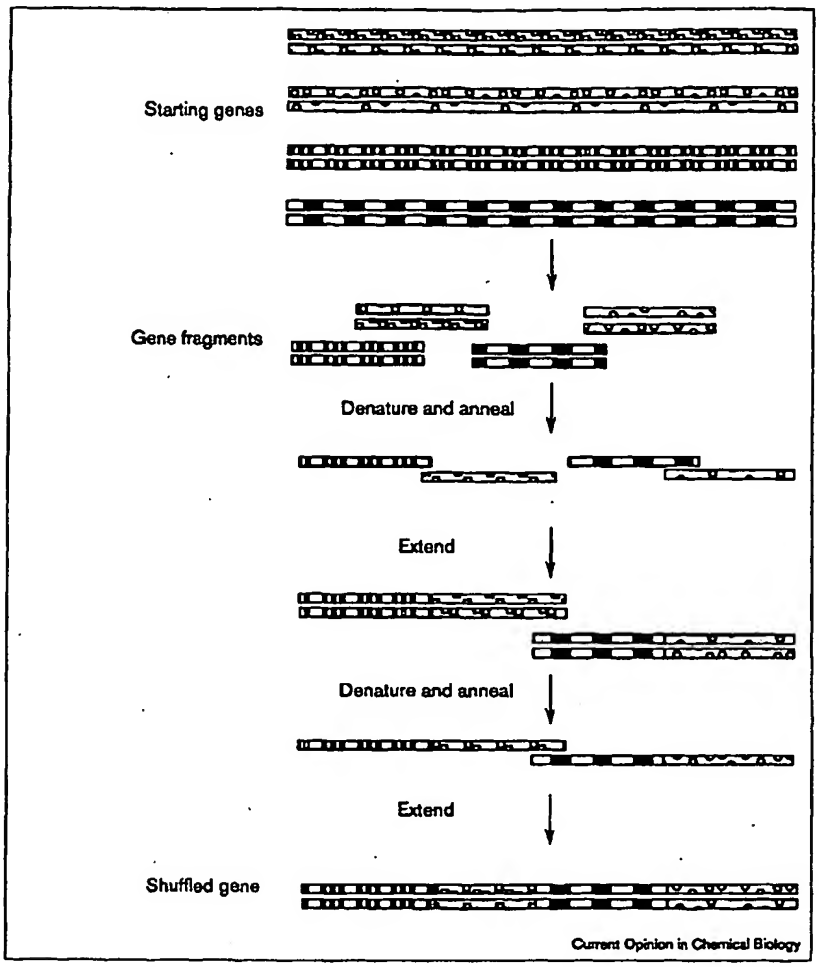
Directed evolution effectively performs the complex computations required to determine the effects of changes in sequence on catalytic function. In addition to the active-site geometry, the impact of sequence changes on protein expression, stability and folding, and interactions with other host proteins and small molecules are all simultaneously considered simply by directly measuring the activity of the mutant enzymes or metabolic pathways.

The best evolutionary strategies are likely to be those that most closely mimic natural ones: in three billion years, not only have individual genes evolved, but the evolutionary process itself has been optimized [6]. Those algorithms that are best at searching through the possible combinations of nucleotides for sequences with biological function have been preserved along with the sequences whose evolution they have facilitated. Recombination is such a mechanism, found universally in biological systems. Genetic algorithms and other computer simulations of simple evolving systems that incorporate the ability to recombine information are more powerful and evolve more rapidly than those which do not [6–9].

Incorporation of recombination into a method for directed evolution of single genes (known as 'DNA shuffling' or 'molecular breeding') was developed recently [10]. In this method, a population of mutant genes (rather than just one) are selected on the basis of their containing beneficial mutations, thus making them appropriate as parents for the next cycle. The genes are randomly fragmented, then reassembled by recombination with each other. The process is shown schematically in Figure 1. As well as accelerating the *in vitro* evolutionary process [10–12], the shuffling reaction is extremely flexible: many different pieces of genetic information may be included if they are available (see Figure 2; [13]). For example, Liu *et al.* [14] included degenerate oligonucleotides in their shuffling reaction in order to randomize amino acids believed, through structural studies, to be important for the substrate specificity of a tRNA synthase. Interestingly, only one of the five targeted residues was mutated in the enzyme showing highest activity against the new substrate.

Figure 1

In vitro recombination by DNA shuffling. Genes are fragmented and then reassembled by a reaction in which homologous fragments act as primers for each other.



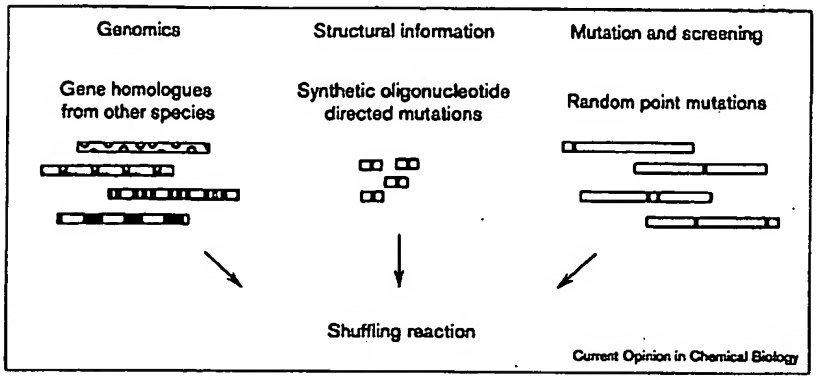
Many examples of successful directed evolution using DNA shuffling have been reviewed recently [15•,16•]. Last year, several additional formats were described for the *in vitro* [17,18] or *in vivo* [19] shuffling of genes. While these methods have not been thoroughly compared, they rely on the same underlying principle that the most efficient way to explore all of the possible combinations and permutations of sequences (i.e. sequence space) is by recombination of active variants.

Screening and selection

Natural evolution measures the fitness of variants by their ability to survive. In some cases, there are genetic selections that can be employed to make a cell's growth dependent on a particular improved function. Schellenberger's group [20•] recently selected for increased subtilisin production by making a target protein the sole source of nitrogen, performing the growth in hollow fibres to prevent cross-feeding. As an artificial selection system, phage display has been used to identify proteins that bind specific ligands. Catalytic proteins displayed on phage have also been selected, either by making infectivity dependent on formation of a covalent intermediate [21••], or by requiring enzyme activity to release the phage from a solid matrix [22•]. Both of these methods only require a single catalytic event, so are unsuitable for quantitative measurements.

Directed evolution has been used to enhance lipase enantioselectivity. Lipases accept a wide variety of non-natural esters, so lipases that are able to discriminate between stereoisomers allow the production of optically pure compounds useful in pharmaceutical and fine chemical manufacture. One group used a microtitre-based absorbance assay in which the esterase activity of lipase variants was measured against the *R* and *S* forms of *p*-nitrophenyl 2-methyldecanoate. Four cycles, testing 1,000 lipase mutants per cycle, increased the enantioselectivity from 2% enantiomeric excess (ee) to 81% ee in favor of the *S* configuration [23]. A second group evolved an

Figure 2



The shuffling reaction is extremely flexible. Positive variants resulting from random mutation and selection can be recombined with sequence information obtained computationally. Genomics allow the inclusion of related genes from other species and structural information can be used to design synthetic oligonucleotides for making specific changes or to randomize targeted regions of a protein.

enzyme to hydrolyze an ester for production of an intermediate in epithilone synthesis. The initial screen for this enzyme was performed by including both the enzyme substrate and a pH indicator in agar plates. Bacterial colonies expressing an enzyme able to hydrolyze the ester were identified by a change in the colour of the indicator, since acid is released when esters are hydrolyzed. Colonies selected by this screen were then picked and tested for their biotransformation activity and stereoselectivity by measuring the optical rotation of the products [24•]. While individual screens will always depend on the reactions being catalyzed, this strategy of tiered screening in which a primary, relatively inaccurate assay is used to select a small number of clones that are then subjected to more detailed analysis (see Figure 3) is an extremely powerful general technique.

It is also possible to perform an entire selection *in vitro*. As an example, a library of genes was transcribed and translated in compartments formed in a water/oil emulsion. Active DNA methyltransferase *Hae*III enzymes methylated the genes that encoded them, thereby protecting the DNA from subsequent *Hae*III digestion [25••]. By using such a system, cloning or transformation of the library is not required, so much larger libraries can be screened. Further advances such as coupled reactions leading to gene modification and sorting of intact compartments based on fluorescence would help make *in vitro* enzyme production and testing a very powerful methodology.

Using natural diversity

In addition to developing screening strategies that allow greater numbers of mutants to be screened, directed evolution can be optimized by building protein libraries that contain the maximum number of active (and different) members. Until this year, single genes were used as starting points for DNA shuffling and variants, arising by point mutation, were very similar in sequence to the parent gene. Another approach uses principles similar to those of the mammalian immune system. Antibodies capable of binding essentially any epitope with nanomolar association constants are generated by recombinination between a few thousand sequences, followed by 'affinity maturation' by point mutation [26]. Enzyme catalysis results from binding to and stabilizing the relevant transition-state analogue [27], so it should be possible to harness such a system to produce enzymes [28]. Antibodies have evolved as rigid binding molecules, however, and catalytic antibodies are selected solely by their abilities to bind transition-state analogues rather than other enzymatically essential functions such as substrate binding and product release. They are thus generally much less active as catalysts than proteins that have evolved as enzymes.

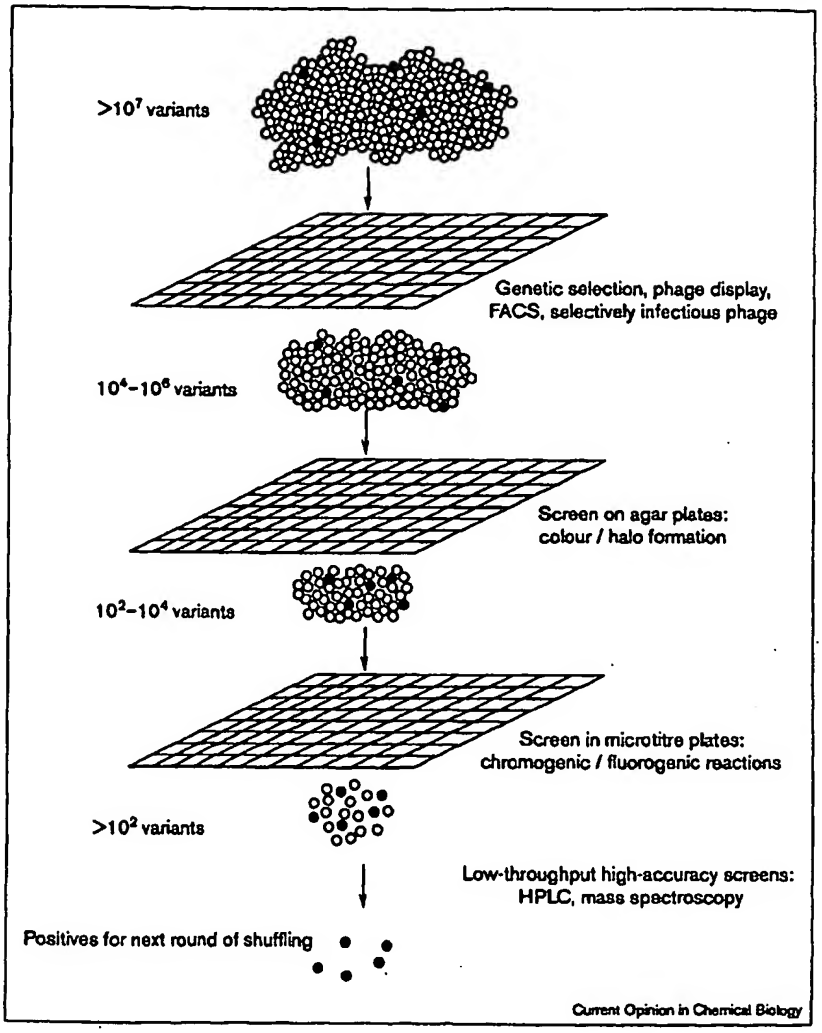
Instead of trying to turn antibodies into catalysts, DNA shuffling can be used to mimic the immune system's incredibly powerful diversity-generating process, by recombining genes with one another. In the first example of 'DNA family shuffling', four different β-lactamase genes were shuffled together to produce a chimera with 270-fold greater resistance to moxalactam than the best parental enzyme [29•]. The chimeric enzyme produced in this experiment differed from each parent by at least 100 amino acids (Figure 4), yet was still a fully functional cephalosporinase. Like antibody 'diversity' regions, sequences that occur in naturally existing enzymes have already been tested for their ability to function within the context of the protein's overall structure. Recombining natural blocks of sequence with each other allows a broad region of functional sequence space to be sampled sparsely.

Protein chimeras may differ dramatically from all their parents

Where an active site lies at the interface between folding subdomains, exchanging these subdomains will alter the shape of the active site. For example, swapping domains between coagulation factor X and trypsin produced a serine protease with broadened substrate selectivity [30•]. The activities of chimeric enzymes are often not predictable simply by comparing those of the parent enzymes,

Figure 3

Tiered screening. Variants are tested by a series of assays that are successively more accurate and more time- and labour-intensive. It is important to ensure that the higher capacity assays correlate well with the desired final activity. FACS, fluorescence-activated cell sorting; HPLC, high-pressure liquid chromatography.

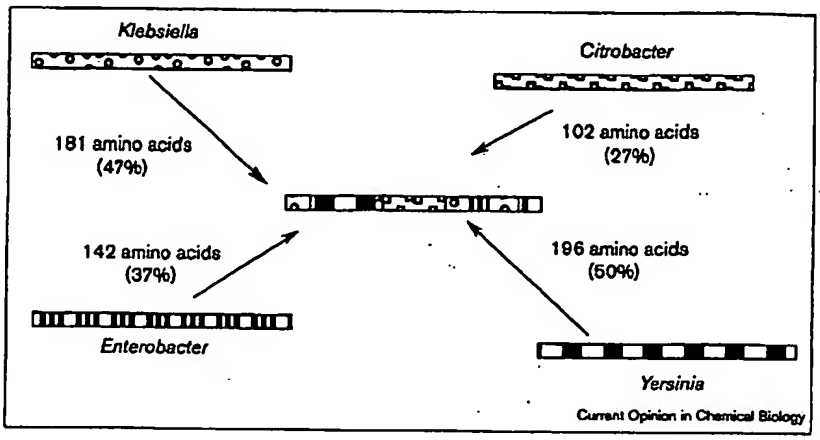


as was found for chimeras between two human blood group glycosyl transferases that were shown to be functionally interconvertible by changing only four amino acids. Parental enzyme A transfers *N*-acetylgalactosamine to a disaccharide acceptor, whereas enzyme B transfers galactose. Replacement of Arg176 in enzyme A with the Gly176 of enzyme B resulted not in increases in B-like activities, but in a fourfold higher k_{cat}/K_M for the enzyme A substrate (i.e. *N*-acetylgalactosamine) [31••].

Altered substrate specificities have also been produced by random recombination of sequences followed by screening. Biphenyl dioxygenases initiate the degradation of polychlorinated biphenyls, and their congener substrate specificities are determined by the large terminal subunit [32]. DNA shuffling of two such dioxygenases produced chimeras with a different substrate range from either parent, enhanced degradation of biphenyl compounds and even novel oxygenation activity for single aromatic hydrocarbons [33,34••].

Random chimeras have also been made *in vivo* between two staphylococcal lipases with differing chain-length selectivities and phospholipase activities. Novel enzymes were found that possessed both combinations of and absolute levels of these activities that differed from both parents in ways that were often surprising [35•]. For example, one chimera in which a block comprising 20% of the enzyme with no chain-length selectivity was incorporated into the enzyme with a strong preference for short-chain fatty acids unexpectedly resulted in an enzyme with twofold increased activity (relative to the best parent) against the long-chain ester *p*-nitrophenyl palmitate.

Figure 4



Mutational distances of chimeric β-lactamase with 270-fold improved moxolactamase activity from its four parents. Distances from each parent are given in number of amino acids, and in the percentage of residues that this represents. The chimera differs by 102 amino acids, that is 27% of positions, from its closest parent (the *Citrobacter* enzyme). It would not be possible to make 102 random changes without inactivating the enzyme. Thus recombination of natural diversity allows functional sequence space to be sampled much more broadly and sparsely than sequential point mutations from a single starting sequence.

Recursive cycles of shuffling using multiple parents has been performed by Christians *et al* [36••]. By recombining two Herpes Simplex Virus thymidine kinase genes and robotically screening for variants that were better able to phosphorylate the therapeutic nucleotide analogue AZT, the concentration of AZT required to inhibit cell growth was reduced 32-fold relative to that required with the best parent. The resulting enzyme was a chimera that had undergone ten cross-over events between the two parental genes, and had also accumulated five point mutations, leading to a protein differing by 22 amino acids from the closest parent. The process of recombination between different but functional parents to make large changes in sequence, coupled with point mutagenesis to fine-tune the activity of the protein, is highly analogous to the process of antibody generation and maturation.

Directed searches for novel protein activities

Although it is possible to modify the physical properties of an enzyme, such as thermostability or activity in organic solvent, by screening for sequential improvements in these properties [37–39], modification of one property by single point mutations can often compromise another desired characteristic [40•]. From the results discussed above, we would predict that by recombining sequences found in nature, it should be possible to discover enzymes possessing all combinations of properties of the individual parents, as well as improvements over any of the parents.

The classification of enzymes into superfamilies that appear to be related by a common chemical strategy for stabilizing the transition state for the formation of a reactive intermediate suggests a mechanism by which nature may evolve novel catalytic functions [41]. Is it possible to make such changes in the laboratory? It may not be possible to make a graded change from one reaction to another. By making structural comparisons between an oleate desaturase and an oleate hydroxylase, Broun *et al.* [42••] have shown that four amino acid changes in the desaturase can convert it to a hydroxylase and changing six residues in the hydroxylase result in desaturase activity. Making these changes by sequential point mutagenesis would not be possible because the single or double mutants do not possess intermediate activities. The exchange of blocks of amino acids made possible by family shuffling, however, offers a possible route to completely novel substrate specificities. Enzyme libraries constructed from relatively small families of homologous genes are likely to contain not only a range of substrate specificities, but also a variety of physical properties and even new catalytic activities. These libraries can then serve as sources of diversity themselves, providing the starting points for further directed evolution in many different directions.

Conclusions

By copying the natural mechanisms by which even existing diversity can be recombined, DNA shuffling can be used to generate high-quality libraries of novel proteins. Chimeras between naturally occurring enzymes that differ by only a few amino acids often possess activities that are significantly different from their parents. By screening these libraries using innovative high-throughput assay techniques, it is possible to identify enzymes with new catalytic functions and physical properties.

Acknowledgements

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